

Human apoA-I/C-III/A-IV gene cluster transgenic rabbits: effects of a high-cholesterol diet

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Abstract We have generated transgenic rabbits that express the entire human apoA-I/C-III/A-IV gene cluster. As in humans, h-apoA-I and h-apoC-III were expressed in liver and intestine, whereas h-apoA-IV mRNA was detected in intestine only. Transgenic rabbits had significantly higher plasma total cholesterol, HDL-cholesterol and total phospholipid concentrations than non-transgenic littermates. In contrast to similar transgenic mice previously generated, which have gross hypertriglyceridemia, triglyceride concentrations were only moderately raised in transgenic rabbits. Plasma and HDL from transgenic rabbits were more effective than those from controls in promoting cholesterol efflux from cultured hepatoma cells. They had lower LCAT, lower CETP and higher PLTP activities than non-transgenic littermates. Cholesterol-feeding produced major increases in plasma lipids. The qualitative response to the diet was not modified by cluster expression. Human apoA-I concentration was halved by cholesterol-feeding, whereas h-apoC-III and h-apoA-IV concentrations were not significantly altered. Cholesterol efflux from hepatoma cells to plasma and HDL was not altered by the diet. Since lipoprotein metabolism of rabbits closely resembles that of humans, human apoA-I/C-III/A-IV transgenic rabbits may provide a reliable model for studies of the transcriptional regulation of the cluster, and for evaluating the effects of different agents on the expression of the three genes. © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

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1. Introduction

The genes coding for human apolipoproteins (apo) A-I, C-III, and A-IV are tandemly organized in a cluster located on the long arm of chromosome 11q23-q24 (h-apoA-I/C-III/A-IV). The tissue-specific expression of the three genes is primarily controlled at the level of transcription, which is

coordinately regulated by positive and negative DNA elements spread throughout the cluster sequence [1,2].

ApoA-I, apoC-III and apoA-IV make essential contributions in lipid transport and homeostasis. Mutations in the cluster gene sequence have major consequences in lipoprotein remodeling, and have been associated with several dyslipoproteinemias and premature coronary artery disease [3].

Much effort has been directed to the study of transgenic animals expressing the h-apoA-I, h-apoC-III or h-apoA-IV genes, and these have provided new insights into the function of each apolipoprotein and the mechanism of atherogenesis [4–10]. However, none of these models is suitable for analysis of the coordinated expression of the three genes. For that reason, we have previously generated transgenic mice expressing the entire gene cluster h-apoA-I/C-III/A-IV [11].

Lipoprotein metabolism and atherogenesis in rabbits resemble their human counterparts more closely than do those in mice. Rabbits, like humans and unlike mice, express cholesteryl ester transfer protein (CETP), which plays a key role in lipoprotein remodeling. In addition, in rabbits the concentration and composition of apoB-containing lipoproteins are similar to those in humans. Furthermore, in the rabbit hepatic apoB100 and intestinal apoB48 synthesis pattern resembles that of humans [12]. Therefore, in the present work we have generated and characterized transgenic rabbits expressing the h-apoA-I/C-III/A-IV cluster, and have studied the effects of a high-cholesterol diet in the animals.

2. Materials and methods

2.1. Animals

A transgene containing 8.3 kb of the 5' region of apoA-I, the entire 17-kb h-apoA-I/C-III/A-IV gene cluster, and 7.5 kb of the 3' region of apoA-IV was micro-injected into fertilized oocytes from New Zealand White rabbits. We have previously used the same transgene to produce h-apoA-I/C-III/A-IV transgenic mice [11]. Three different transgenic lines were established (lines 8, 15 and 23). All procedures involving animal handling and care were conducted in accordance with the guidelines of the French Commission de Génie Génétique.

2.2. Cholesterol-feeding experiment

Transgenic rabbits from line 8 and non-transgenic littermates aged 3 months were fed either a high-cholesterol (0.5% w/w) or a chow diet

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for 12 weeks. Each of four groups was composed of 4 males and 4 females.

2.3. Northern blots

Total RNA was extracted from different tissues (liver, small intestine, duodenum, spleen, kidney, brain, lung and heart) with RNA-plus (Q-BIOgene). Northern blot assays were performed with cDNA probes for each human apolipoprotein as previously reported [11]. Human β -actin (Clontech) or 18S rRNA probes were used as internal controls to normalize apolipoprotein signals.

2.4. Apolipoprotein, lipid and lipoprotein analyses

Human apoA-I and apoC-III were quantified by immunoelectrophoresis using specific polyclonal antibodies (Hydragels SEBIA). Human apoA-IV was measured by a sandwich ELISA [13]. Rabbit apolipoproteins did not cross-react. Plasma lipids (total and free cholesterol, triglycerides and phospholipids) and HDL-cholesterol were measured colorimetrically (Boehringer Mannheim). To quantify HDL-cholesterol, apoB-containing lipoproteins were precipitated with sodium phosphotungstate/magnesium chloride (Boehringer Mannheim).

Lipoproteins from pooled plasma samples were separated into density (d) fractions by sequential isopycnic ultracentrifugation as described [14].

2.5. Cholesterol efflux experiments

Cellular cholesterol efflux was determined using Fu5AH rat hepatoma cells as previously described [15]. The cells were incubated with either 5% diluted serum or HDL fraction (obtained by ultracentrifugation) at a protein concentration of 50 μ g/mL. All efflux values presented are averages of three determinations.

2.6. Enzyme and lipid transfer protein activities

Lecithin:cholesterol acyltransferase (LCAT) activity was determined by use of the exogenous proteoliposome substrate method [16]. CETP and phospholipid transfer protein (PLTP) activities were measured using the methods described by Lagrost [17] and Damen [18], respectively.

2.7. Statistical analyses

Data are expressed as means \pm standard deviation (S.D.). Statistical analysis was performed with the StatView 4.5 program. Differences between groups were analyzed by two-way ANOVA and were considered to be significant if $P < 0.05$.

3. Results

3.1. Characterization of human apoA-I/C-III/A-IV transgenic rabbits

Human apoA-I/C-III/A-IV expression. Southern blot analyses revealed that the three established transgenic lines (8, 15 and 23) contained the entire h-apoA-I/C-III/A-IV cluster and its flanking sequences (not shown). In transgenic rabbits from the three lines, h-apoA-I and h-apoC-III mRNAs were present in the liver and intestine, whereas h-apoA-IV mRNA was detected only in the intestine, corresponding to the human expression pattern. No expression of the human apolipoproteins was observed in other tissues that were analyzed. Results for a representative transgenic line are shown in Fig. 1.

Human apolipoprotein and lipid concentrations. Human apolipoprotein and lipid concentrations were assayed in 3-months-old transgenic rabbits from the three established lines. The lipid profile of each line was compared with that of age-matched non-transgenic littermates.

The three human apolipoproteins were present in plasma from all transgenic rabbits (Table 1). Transgenic rabbits had significantly raised plasma lipid concentrations relative to those in non-transgenic littermates (Table 1). Both total

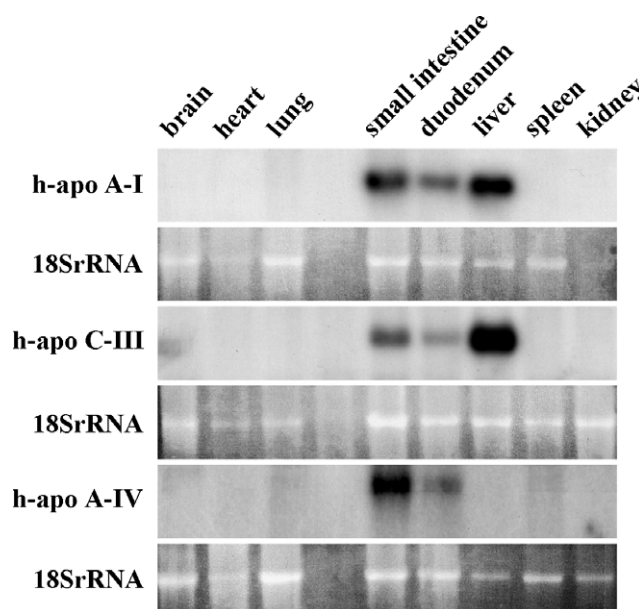


Fig. 1. Northern blot analysis of human apolipoprotein (apo) A-I, apoC-III and apoA-IV mRNA in a representative human apoA-I/C-III/A-IV transgenic rabbit (line 8). Each membrane was hybridized with two radioactive probes: h-apoA-I, h-apoC-III, or h-apoA-IV, and 18S rRNA. Expression was analyzed in brain, heart, lung, small intestine, duodenum, liver, spleen and kidney.

cholesterol and HDL-cholesterol concentrations were significantly greater in transgenic rabbits, but there was no significant difference in the concentration of non HDL-cholesterol. Phospholipids were also greatly increased. Triglyceride concentrations were moderately raised in h-apoA-I/C-III/A-IV transgenic rabbits relative to control littermates.

3.2. Effects of a high-cholesterol diet

In order to assess the effect of h-apoA-I/C-III/A-IV expression on lipoprotein metabolism, transgenic rabbits from line 8 and control littermates were fed a high-cholesterol diet for 12 weeks.

Apolipoprotein expression. Human-apoA-I mRNA in liver and intestine were reduced by approximately 50% in the cholesterol-fed rabbits relative to transgenic rabbits fed the chow diet. Human apoC-III expression was also reduced in liver of cholesterol-fed rabbits, but was not affected in the intestine. Finally, h-apoA-IV mRNA was not significantly modified in intestine by the diet, while it remained non-detectable in liver (Table 2).

Human apolipoprotein and lipid concentrations. Human apoA-I concentration in the plasma of transgenic rabbits was significantly diminished by the high-cholesterol diet. In contrast, h-apoC-III and h-apoA-IV concentrations were not significantly altered. Surprisingly, h-apoC-III levels were modified in transgenic rabbits after 12-weeks on the chow diet (compare data from Tables 1 and 3). The high-cholesterol diet caused major increases in plasma total cholesterol, triglyceride and phospholipid concentrations in both transgenic and control rabbits, but the lipid concentrations remained significantly higher in transgenics. Significant differences resulted from both genetic and diet effects, as well as from the interaction of both factors. Triglyceride concentrations showed great variability among the four groups. Furthermore, in accordance with the

Table 1
Plasma lipid and human apolipoprotein concentrations (mg/dL) in control and transgenic rabbits

Strain	Total cholesterol	Triglyceride	HDL-C	Phospholipid	ApoA-I	ApoC-III	ApoA-IV
Control (<i>n</i> = 69)	50 ± 17.4	42 ± 16.3	30 ± 8.4	84 ± 25.9	ND	ND	ND
Tg8 (<i>n</i> = 22)	81 ± 20.7*	66 ± 33.2 [†]	56 ± 16.8*	156 ± 37.6*	146 ± 20.3	0.6 ± 0.36	7.5 ± 1.73
Tg15 (<i>n</i> = 19)	66 ± 17.0 [†]	60 ± 27.1 [†]	47 ± 12.0 [§]	116 ± 40.6 [§]	119 ± 19.4	1.1 ± 0.44	5.4 ± 2.09
Tg23 (<i>n</i> = 11)	78 ± 18.5 [†]	52 ± 13.7	49 ± 11.9 [†]	139 ± 37.3 [§]	128 ± 18.5	1.2 ± 0.59	4.9 ± 0.68

Data are means ± S.D. ND indicates not determined.

* $P < 0.0001$, [§] $P < 0.01$, and [†] $P < 0.05$ vs. non-transgenic control rabbits.

Table 2
Abundance of apoA-I, apoC-III and apoA-IV liver and intestinal mRNAs in transgenic rabbits from line 8 on chow and cholesterol-rich diets

Organ	Diet	ApoA-I	ApoC-III	ApoA-IV
Liver	Chow	1.7 ± 0.47	3.5 ± 0.61	ND
	Cholesterol	0.9 ± 0.26**	2.2 ± 0.59**	ND
Intestine	Chow	1.4 ± 0.47	0.9 ± 0.28	1.3 ± 0.38
	Cholesterol	0.8 ± 0.16*	1.0 ± 0.25	1.2 ± 0.48

Data are means ± S.D., *n* = 8. ND indicates not detected.

Values are reported as arbitrary units normalized to β-actin mRNA levels.

* $P < 0.05$, ** $P < 0.01$ vs. chow diet fed rabbits.

Table 3
Plasma lipid and apolipoprotein concentrations (mg/dL) in control and transgenic rabbits from line 8 on chow and cholesterol-rich diets

	Diet	Total cholesterol	Triglycerides	Phospholipid	ApoA-I	ApoC-III	ApoA-IV
Control	Chow	48 ± 27.1a	18 ± 18.6a	66 ± 18.7a	ND	ND	ND
	Cholesterol	1225 ± 291.4b	197 ± 103.5b	543 ± 108.8b	ND	ND	ND
Tg8	Chow	92 ± 35.6c	186 ± 112.8b	197 ± 45.0c	190 ± 47.7a	2.0 ± 0.79a	7 ± 3.0a
	Cholesterol	1931 ± 51.1d	921 ± 619.6c	1424 ± 361.3d	116 ± 21.7b	2.5 ± 1.18a	5 ± 2.4a
Two-way ANOVA	Genetic effect	<0.0001	0.0003	<0.0001	ND	ND	ND
	Diet effect	<0.0001	0.0003	<0.0001	ND	ND	ND
P-values	Interaction	<0.0001	0.02	<0.0001	ND	ND	ND

Data are mean ± S.D., *n* = 8. ND indicates not determined.

Values in each column with different letters differ, $P < 0.05$.

observed modification of the h-apoC-III concentration on the same diet (see above), plasma triglyceride concentrations in transgenic rabbits fed the chow diet changed significantly during the 12-weeks experiment.

Lipid distribution. Plasma lipoproteins were isolated by sequential ultracentrifugation from two pools of plasma for each group of rabbits, and their lipid and apolipoprotein compositions were analyzed. The effects of the high-cholesterol diet on lipid distribution were similar in transgenic and control rabbits. While the high-cholesterol diet greatly raised non-HDL cholesterol concentration, little change was observed in HDL-cholesterol. Therefore, the percentage of HDL-cholesterol in plasma became much lower than during the chow diet (Fig. 2A). A similar pattern was obtained for the phospholipid distribution (data not shown). In transgenic rabbits, the high-cholesterol diet raised triglycerides mainly in very low density (VLDL) and intermediate density (IDL) lipoproteins, and reduced it in HDL particles (Fig. 2B).

LCAT, PLTP and CETP activities. During chow diet feeding, LCAT activity was significantly lower in the transgenic rabbits than in control littermates ($21 \pm 6.6\%$ vs. $38 \pm 13.0\%$, $P < 0.01$). The high-cholesterol diet caused a 10-fold decrease of LCAT activity in both transgenic and control rabbits ($2 \pm 2.8\%$ vs. $3 \pm 1.9\%$, NS). In conformity with this, the percentage of cholesteryl ester to total cholesterol was lower in transgenic than in control rabbits during the chow diet

($75 \pm 8.8\%$ vs. $95 \pm 5.5\%$), and it diminished during the cholesterol rich diet in both the transgenics and controls ($51 \pm 13.6\%$ vs. $68 \pm 2.7\%$). CETP activity was also lower in transgenic rabbits than in the controls during the chow diet ($10 \pm 7.4\%$ vs. $26 \pm 7.9\%$, $P < 0.01$). The high-cholesterol diet increased CETP activity in transgenics and controls by 8- and 3-fold, respectively ($80 \pm 1.6\%$ vs. $82 \pm 1.6\%$, NS).

In contrast, PLTP activity was higher in the transgenic than in the control animals on a chow diet ($2.82 \pm 0.29 \mu\text{mol/mL}$ vs. $1.70 \pm 0.44 \mu\text{mol/mL}$, $P < 0.001$). During the high cholesterol diet, PLTP activity decreased in both groups (1.29 ± 0.30 vs. $0.32 \pm 0.10 \mu\text{mol/mL}$, $P < 0.006$).

Cholesterol efflux. Independently of the diet, plasma from transgenic rabbits was more effective than plasma from control rabbits in promoting cholesterol efflux from cultured hepatoma cells (Fig. 3). The high-cholesterol diet increased cholesterol efflux to control rabbit plasma ($P < 0.01$), but not to transgenic rabbit plasma. When we measured the capacity of the HDL fraction to induce efflux of cholesterol, we observed that it was greater with HDL from transgenic rabbits than with HDL from control animals. This difference was greater during the chow diet ($8.32 \pm 0.51\%$ vs. $3.44 \pm 0.42\%$, $P < 0.001$) than during the high cholesterol diet ($4.55 \pm 0.68\%$ vs. $2.98 \pm 0.27\%$, $P < 0.01$). Interestingly, these values were closely correlated ($r = 0.968$, $P < 0.0001$) with the phospholipid content of the HDL fractions ($122.3 \pm 4.16 \text{ mg/dL}$ vs. $25.4 \pm 5.02 \text{ mg/dL}$ for

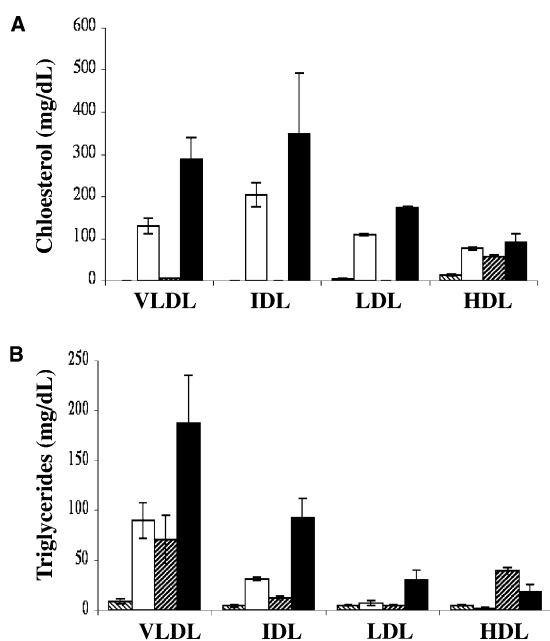


Fig. 2. Lipid concentrations in lipoproteins of control and human apoA-I/C-III/A-IV transgenic rabbits from line 8 fed chow or high-cholesterol diets. Lipoproteins were isolated by sequential preparative ultracentrifugation of two pools of plasma from each group of rabbits. Each pool was prepared from 4 rabbits. Lipids were quantified by enzymatic methods. (A) Cholesterol. (B) Triglycerides. Dashed bars to left: control rabbits fed chow diet; open bars: control rabbits fed cholesterol-rich diet; dashed bars to right: transgenic rabbits fed chow diet; filled bars: transgenic rabbits fed cholesterol-rich diet. Values for each bar correspond to means \pm S.D.

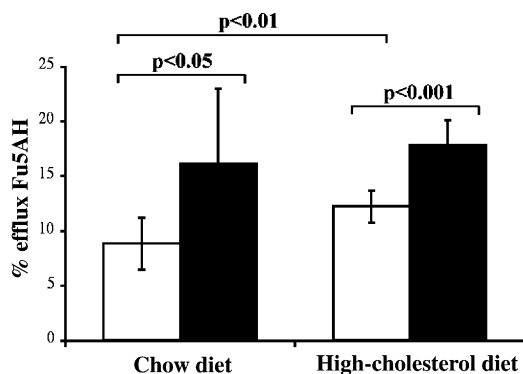


Fig. 3. Efflux of [3 H]cholesterol from Fu5AH to plasma from control (open bars) and human apoA-I/C-III/A-IV transgenic (filled bars) rabbits from line 8 fed chow or high-cholesterol diets. Data are from a representative experiment with triplicate wells. Values are expressed as means \pm S.D. Statistically significant differences between control and transgenic rabbits fed the same diet and between control rabbits fed a chow or a high-cholesterol diet are indicated in the figure.

transgenic and control rabbits on the chow diet, and 80.7 ± 11.97 mg/dL vs. 38.9 ± 8.33 mg/dL on the high cholesterol diet).

4. Discussion

The present paper reports the first successful production of rabbits expressing the entire human apoA-I/C-III/A-IV gene

cluster. The tissue specific expression of the human apoA-I, apoC-III and apoA-IV genes in the rabbits was the same as that in humans. Hence, this new strain of transgenic rabbits may provide a good model for studies of the transcriptional regulation of the human cluster.

The effects of expression of the human cluster on plasma lipoproteins in rabbits differed from those in our human apoA-I/C-III/A-IV gene cluster transgenic mice [11]. The most striking difference was in triglyceride concentrations. While mice developed gross hypertriglyceridaemia, 3-month old cluster rabbits showed only a modest rise in plasma triglycerides. This difference was probably related to their lower human apoC-III concentrations. Nevertheless, plasma triglyceride and human apoC-III concentrations were surprisingly increased in 7-month-old transgenic rabbits fed a chow-diet, although they remained lower than in cluster mice. Both transgenic species developed an increase in plasma HDL-cholesterol. However, while transgenic mice showed a major rise in non-HDL-cholesterol, this was not significantly modified in transgenic rabbits.

As would be expected, cholesterol-feeding increased plasma lipid concentrations in both transgenic and non-transgenic rabbits. Lipid levels remained higher in the transgenics than in the non-transgenics throughout. The changes in the distributions of lipids among the major density fractions of lipoproteins in both groups were consistent with an accumulation of circulating chylomicron remnants. Thus, cluster expression appeared to have little effect on the qualitative nature of the response to the diet.

Cholesterol-feeding had no effects on the expression levels of the human apoC-III and apoA-IV genes in small intestine, but reduced those of human apoA-I in liver and intestine and of human apoC-III in liver. The decrease in apoA-I expression was accompanied by a reduction of plasma apoA-I concentration, while concentrations of human apoC-III and human apoA-IV were unchanged. The failure of apo human C-III to decrease despite a reduction of human apoC-III expression in liver during cholesterol-feeding may be attributable to the fact that the fractional rate of catabolism of C apolipoproteins has been shown to be a function of the efficiency of clearance of triglyceride-rich lipoproteins and their remnants [19], the mechanisms for which may have been saturated in the transgenics during cholesterol-feeding. In cluster transgenic mice, feeding a high fat-high cholesterol diet resulted in an increase in the expression of the three human apolipoprotein genes in intestine and a decrease in the expression of human apoA-I gene in liver. The plasma concentrations of the three human apolipoproteins were significantly greater in transgenic mice fed the atherogenic diet than in chow fed mice [20]. Although we do not have a clear explanation for this discrepancy between cluster rabbit and mouse models, there are probably differences between the properties of mouse and rabbit transcriptional factors involved in the gene cluster transcription, leading to modified affinities for the human gene cluster regulatory sequences. Furthermore, differences in the compositions of the diets employed in each study may also have contributed to the different apolipoprotein expression levels.

On the chow diet transgenic rabbits had lower LCAT and CETP activities, and higher PLTP activities, than their non-transgenic littermates. During cholesterol-feeding, qualitatively similar changes were observed in all three measurements in transgenics and non-transgenics, LCAT and PLTP activities

decreasing, and CETP activity increasing. The mechanism of these effects cannot be determined from the present data.

Preliminary experiments performed with groups of 8 animals demonstrated that the high cholesterol diet caused the development of extensive aortic atherosclerosis in both transgenic and control rabbits (data not shown). No significant difference was found between the two groups of rabbits in the total surface area of the lesions. However, as the extent of atherosclerosis varied greatly between individual animals of each group, a larger sample size will be needed to resolve this issue.

Although cholesterol efflux from cultured hepatoma cells to plasma and isolated HDLs was greater when transgenic samples were used, cholesterol-feeding increased these values only in the controls. However, cholesterol efflux from Fu5AH cells is a reliable guide only to cholesterol efflux stimulated by SR-BI, and further analyses should be performed with J774 cells stimulated by cAMP in order to provide a better indication of the transfer of cholesterol from the ABCA1 receptors [21].

In summary, we have developed the first strain of rabbits expressing the entire human apoA-I/C-III/A-IV gene cluster. Although further work will be needed to characterize the animals in greater detail, our results thus far suggest that they may provide a good model for future work on the transcriptional regulation of the human cluster, and for evaluating the effects of drugs, hormones and other agents on the expression of the three genes.

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